EXHIBIT 16

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Clinical Protocol

Phase I Study of Immunotherapy of Hepatic Metastases of Colorectal Carcinoma by Direct Gene Transfer

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SCHEMA

Prior to discussing protocol entry with the patient, call the Randomization Center to insure that a place on the protocol is open to the patient.

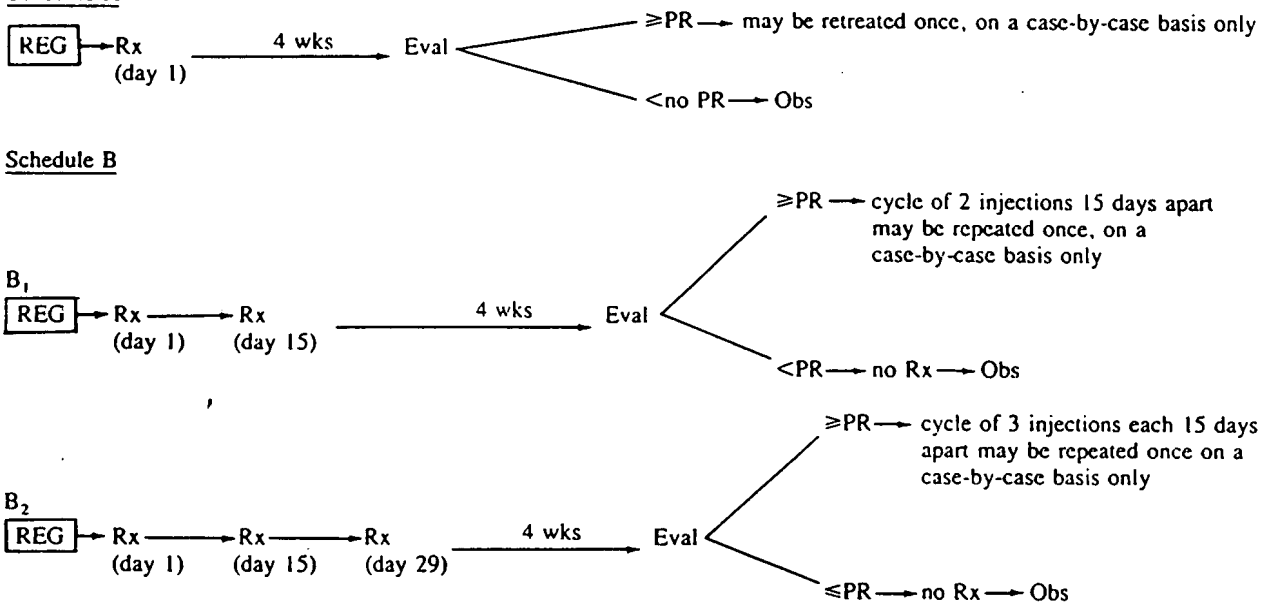
Escalating doses will be evaluated. Groups of patients will receive either escalating doses of an intralesional injection of the test agent into one metastatic lesion in the liver in a single session (Schedule A) or repeated injections at the same dose (Schedule B).

Schedule A

Group	*Number of patients*	*μg of DNA*
A-1	3	10 μg day 1 only
A-2	3	50 μg day 1 only
A-3	3	250 μg day 1 only

Schedule B
(Will be initiated after safety of Schedule A at 50 μg has been established)

Group	*Number of patients*	*μg of DNA*
B-1	3	10 μg days 1 and 15
B-2	3	10 μg days 1, 15, and 29



1.0 Background

1.1 Overview

Cancer is a disease in which certain cells grow uncontrolled by the body's normal self-regulatory mechanisms. Traditional chemotherapy seeks to control cancer by killing rapidly dividing cells or by preventing cells from entering cell cycle and dividing. However, a number of nonmalignant cells in the body, such as bone marrow cells and intestinal epithelium cells, are also rapidly dividing and, hence, are highly susceptible to the toxicity of chemotherapy. Doses sufficient to induce remission in the cancer cannot be administered without life-threatening side effects in 5–10% of the patients and the overall mortality from chemotherapy is 0.5%. A therapeutic approach that selectively kills tumor cells with high efficacy would theoretically be far superior to currently available therapies.

The goal of immunotherapy is to stimulate the immune system to recognize and kill cancer cells by modifying the tumor cells or modifying the host response by such mechanisms as expanding the lymphocytes that respond specifically to the antigens on the tumor cells. Immunotherapy has shown promise as an approach to the treatment of malignancy. Indeed, cancers such as melanoma, renal cell carcinoma, and colon adenocarcinoma are responsive to modulation of immune function, because the immune system can be induced to recognize tumor associated and tumor specific antigens in these cells.

Over the last several decades, there have been many attempts to identify tumor-specific antigens that might be the targets for cytotoxic antibodies or cell-mediated immunity. There have been numerous attempts to develop vaccines and monoclonal antibodies directed at one or more preferentially expressed cell surface antigens in a variety of cancers. Overall, tumor vaccines using intact cells or extracts plus adjuvants have given about a 10–20% response rate. Other approaches to immuno-

therapy have involved the administration of nonspecific immunomodulating agents such as Bacillus Calmette-Guerin (BCG), cytokines, and/or adoptive transfer of cytotoxic T cells, which have shown promise in animal models (1–6) and in man (7–10). More recently, molecular genetic interventions have been designed in an attempt to improve the efficacy of immunotherapy.

Nabel and colleagues at the University of Michigan are investigating a novel molecular genetic intervention for human malignancy that enhances the immune response to tumors by in vivo gene transfer. This immunotherapeutic approach based on animal model work (11,12) uses a gene encoding a transplantation antigen, an allogeneic class I major histocompatibility complex (MHC) antigen, HLA-B7, introduced into human tumors in vivo by DNA/lipid complex transfection. The direct intratumoral injection approach is used. Expression of allogeneic MHC antigens on tumor cells stimulates immunity against both the transfected cells as well as previously unrecognized antigens present in unmodified tumor cells. The introduction of an allogeneic MHC gene directly into tumors in vivo has induced partial tumor regressions, as well as specific cytotoxic T cell responses to other antigens.

In a preliminary trial in humans with malignant melanoma, Nabel treated five patients with malignant melanoma. Three patients received three treatments, totalling 0.87 μg of DNA intratumorally and three patients (two additional patients plus one of the original three patients) received cumulative dose of 2.58 μg of DNA via three treatments. No toxicity resulted from this form of treatment and there was no formation of anti-DNA antibody or autoantibody. There was no plasmid DNA detectable in the blood by PCR following gene transfer (tested on days 3–7 post transfection of ~2 pg/mL sensitivity).

Evidence of gene transfer was found on biopsy of the injected tumor. The biopsy samples were analyzed for plasmid DNA, mRNA coding for HLA-B7, and the expression of HLA-B7 protein. In four of the five patients, plasmid DNA and HLA-B7 mRNA were detected within the treated nodules by PCR. HLA-B7 expression was confirmed in all treated nodules by immunohistochemical staining with a monoclonal antibody to the gene product. Two patients, where cell lines were established from the tumor, showed an immune response by lysing autologous tumor cells. One of the five patients had a partial remission which involved cutaneous and visceral disease. (Nabel, et al., PNAS, in press).

These data suggest that tumor cells modified with the HLA-B7 gene not only stimulate CTLs and potentially other immune system cells to recognize tumors expressing HLA-B7, but they may also provide a stimulus to immune cells to eliminate tumor cells at other sites which express tumor associated antigens in association with the patient's own HLA antigens.

Several improvements that may increase the convenience, safety, and efficacy of the procedure have been introduced since the original Nabel studies, including:

—an improved cationic lipid formulation, DMRIE/DOPE*;

—DNA plasmid construction to optimize expression.

The efficacy of transfection was improved for the following reasons: Briefly, a new formulation of cationic lipids has been described recently by Dr. Phillip Felgner (Vical) in which a different cationic lipid, DMRIE (1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide), is utilized with DOPE (dioleoyl phosphatidylethanolamine). This has two properties which make it more suitable for these studies. First, it shows up to 10-fold improved transfection efficiency in vitro compared to the formulation previously used by Nabel. More importantly, this formulation does not aggregate at high concentrations. This characteristic thus allows higher absolute concentrations of DNA and lipid complex to be introduced into experimental animals without toxicity. Because of these properties, it now becomes possible to introduce 100–1000 times more DNA which could allow the study of an expanded dose response gene expression in vivo.

The vector improvements are divided into two categories for this proposal. In the first case, expression of the HLA-B7 vector has been improved by the addition of a consensus translation initiation sequence and removal of an intron. In addition, the inclusion of the β-2 microglobulin gene, with which class I MHC genes normally associate, allows synthesis of the complete histocompatibility molecule, which is composed of these two chains. Ordinarily, these two gene products are co-transported to the cell surface. This is important because some human melanoma cells do not express endogenous β-2 microglobulin, thus limiting their ability to stably express class I on the cell surface. It has been found that the inclusion of the β-2 microglobulin gene on the same plasmid allows for the expression in these otherwise resistant cells and improve expression in other cells, thus overcoming a potential mechanism of resistance. These modifications have been incorporated in the study drug to be used in this submission. The study drug is identical to the study drug fully characterized in Dr. Nabel's RAC submission of June 7, 1993, which was unanimously allowed. This study will investigate the administration of the study drug in malignant melanoma.

1.2 Direct Gene Transfer and Modulation of the Immune System

The utilization of catheter-based gene delivery in vivo provided a model system for the introduction of recombinant gene-specific sites in vivo. Early studies focused on the demonstration that specific reporter genes could be expressed in vivo (13,14). Subsequent studies were designed to determine whether specific biologic responses could be induced at sites of recombinant gene transfer. To address this question, a highly immunogenic molecule, a foreign major histocompatibility complex (MHC), was used to elicit an immune response in the iliofemoral artery using a porcine model. The human HLA-B7 gene was introduced using direct gene transfer with a retroviral vector or DNA lipid complex (12). With either delivery system, expression of the recombinant HLA-B7 gene product could be demonstrated at specific sites within the vessel wall. More importantly, the expression of this foreign histocompatibility antigen induced an immunologic response at the sites of this foreign histocompatibility antigen induced an immunologic response at the sites of genetic modification. This response in-

*DMRIE, 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium; DOPE, dioleoyl phosphatidylethanolamine bromide.

cluded a granulomatous mononuclear cell infiltrate beginning 10 days after introduction of the recombinant gene. This response resolved by 75 days after gene transfer; however, a specific cytolytic T cell response against the HLA-B7 molecule was persistent. This study demonstrated that a specific immunologic response could be induced by the introduction of a foreign recombinant gene at a specific site in vivo. Moreover, this provided one of the fist indications that direct gene transfer of specific recombinant genes could elicit an immune response to the product of that gene in vivo (12).

These studies suggested that the introduction of the appropriate recombinant genes could be used to stimulate the immune system to recognize its product in vivo. In addition, this approach provided a general method for the induction of a specific site in vivo. To determine whether direct gene transfer might be appropriate for the treatment of disease, a murine model of malignancy was developed. Direct gene transfer of an allogeneic histocompatibility complex gene into a murine tumor elicits an immune response not only to the foreign MHC gene but also to previously unrecognzied tumor-associated antigens. These immune responses are T cell-dependent, and these tumor-associated proteins are recognized within the context of the self major histocompatibility complex. In animals presensitized to a specific MHC haplotype, direct gene transfer into established tumors could attenuate tumor growth or, in some cases, lead to complete tumor regression (11). These studies demonstrate that direct gene transfer of foreign MHC genes into tumors have potentially therapeutic effects that may be appropriate for the treatment of malignancy.

1.3 Immunotherapy of Malignancy

In some instances, the immune system appears to contribute to the surveillance and destruction of neoplastic cells, either by mobilization of cellular and humoral immune effectors. Cellular mediators of antitumor activity include MHC-restricted cytotoxic T cells, natural killer (NK) cells (15,16) and lymphokine-activated killer (LAK) cells (17). Cytolytic T cells which infiltrated tumors have been isolated and characterized (18). These tumor infiltrating lymphocytes (TIL) selectively lyse cells of the tumor from which they were derived (3,19). Macrophages can also kill neoplastic cells through antibody-dependent mechanisms (20,21) or by activation induced by substances such as BCG (22).

Cytokines can also participate in the antitumor response, either by a direct action on cell growth or by activating cellular immunity. The cytostatic effects of tumor necrosis factor-α (TNF-α) (23) and lymphotoxin (24) can result in neoplastic cell death. Interferon-γ (IFN-γ) markedly increases class I MHC cell surface expression (25,26) and synergizes with TNF-α in producing this effect (27). Colony stimulating factors such as G-CSF and GM-CSF activate neutrophils and macrophages to lyse tumor cells directly (28), and interleukin-2 (IL-2) activates Leu-19$^+$ NK cells to generate lymphokine activated killer cells (LAK) capable of lysing autologous, syngeneic or allogeneic tumor cells but not normal cells (17,29,30). The LAK cells lyse tumor cells without preimmunization or MHC restriction (31). Interleukin-4 (IL-4) also generates LAK cells and acts synergistically with IL-2 in the generation of tumor specific killer cells (32).

Since most malignancies arise in immunocompetent hosts, it is unlikely that tumor cells have evolved mechanisms to escape host defenses, perhaps through evaluation of successively less immunogenic clones (33). Deficient expression of class I MHC molecules limits the ability of tumor cells to present antigens to cytotoxic T cells. Freshly isolated cells from naturally occurring tumors frequently lack class I MHC antigen completely or show decreased expression (34–38). Reduced class I MHC expression could also facilitate growth of these tumors when transplanted into syngeneic recipients. Several tumor cells lines which exhibit low levels of class I MHC proteins become less oncogenic when expression vectors encoding the relevant class I MHC antigen are introduced into them (39–43). In some experiments, tumor cells which express a class I MHC gene confer immunity in naive recipients against the parental tumor (40,41). The absolute level of class I MHC expression, however, is not the only factor which influences the tumorigenicity or immunogenicity of tumor cells. In one study, mouse mammary adenocarcinoma cells, treated with 5-azacytidine and selected for elevated levels of class I MHC expression, did not display altered tumorigenicity compared to the parent line (44).

The immune response to tumor cells can be stimulated by systemic administration of IL-2 (45) or IL-2 with LAK cells (46,47). Clinical trials using tumor infiltrating lymphocytes are also in progress (48). Recently, several studies have examined the tumor suppressive effect of lymphokine production by genetically altered tumor cells. The introduction of tumor cells transfected with an IL-2 expression vector into syngeneic mice stimulated an MHC class I restricted cytolytic T-lymphocyte response which protected against subsequent rechallenge with the parental tumor cell line (49). Expression of IL-4 by plasmacytoma or mammary adenocarcinoma cells induced a potent antitumor effect mediated by infiltration of eosinophils and macrophages (50). These studies demonstrate that cytokines, expressed at high local concentrations, are effective antitumor agents.

Nabel and coworkers have previously proposed an alternative approach to stimulate an antitumor response, through the introduction of an allogeneic class I MHC gene into established human tumors. The antigenicity of tumor cells has been altered previously by the expression of viral antigens through infection of tumor cells (51–55), or expression of allogeneic antigens introduced by somatic cell hybridization (56,57). Allogeneic class I MHC genes have been introduced into tumor cells by transfection and subsequent selection in vitro. These experiments have produced some conflicting results. In one case, transfection of an allogeneic class I MHC gene (H-2L^d) into an H-2^b tumor resulted in immunologic rejection of the transduced cells and also produced transplantation resistance against the parent tumor cells (58). In another instance, transfection of H-2^b melanoma cells with the H-2D^d gene did not lead to rejection (59), however increased differential expression of H-2D products relative to H-2K may have affected the metastatic potential and immunogenicity of tumor cells (60). The effects of allogeneic H-2K gene expression in tumor cells was examined in another study (61). Several subclones which were selected in vitro and expressed an allogeneic gene were rejected in mice syngeneic for the parental tumor line, however, other subclones did not differ from the parental, untransduced line in generating tumors. This finding suggests that clone-to-clone variation in in vivo growth and tumorigenic capacity may result in other modifications of cells caused by transfection or the subcloning procedure, which affects their tumorgenicity. These types of clonal differences would likely be minimized by transducing a population of cells directly in vivo.

Because the H-2K class I MHC antigen is strongly expressed on most tissues and can mediate an allogeneic rejection response, we chose it in our animal model studies designed to enhance the immunogenicity of tumors in vivo. These studies extended previous efforts to modify tumor cells by developing a system for the direct introduction of genes into tumors by in vivo infection using retroviral vectors or by DNA lipid complex-mediated transfection. This technology can also be used to deliver specific recombinant cytokines into the tumor microcirculation and to understand the immunologic basis for tumor rejection in vivo.

The Department of Diagnostic Radiology, Mayo Clinic-Rochester, has extensive experience in CT and sonographically-guided needle biopsy (see reviews by Charboneau et al. (62) and Charboneau (63). Sonographically-guided biopsy of metastatic lesion in the liver can be carried out with a high degree of accuracy and safety. Overall accuracy was 91% in the biopsy of 126 consecutive masses of various histologic types 3 cm or less in diameter in various anatomic locations. The accuracy increases to 98% for masses 2-3 cm in diameter, the range to which we will restrict delivery of DNA/liposome complex. Current techinques are not limited to this caliber (21–22 gauge needles). It is almost always possible to obtain a core rather than an aspirate of tissue by using a 16–19 gauge needle, needles with cutting ends, and improved biopsy sampling technology. In a study from the Mayo Clinic of 1,000 consecutive CT-guided biopsies, the rate of complication from the use of an 18-gauge biopsy needle was 0.3%, the same as the rate of complication from the use of a 21-gauge needle (64).

This capability allows in one sitting biopsy of the intended target for gene transfer, documentation of metastatic cancer in the lesion, and delivery of DNA/liposomal mixture to the targeted nodule(s). Virtually any region of the liver can be biopsied with safety. For purposes of this proposal, nodules 2–4 cm in size that are easily accessible will be selected to further ensure successful target injection and to reduce the possibility of morbidity.

One advantageous feature of sonographically-guided delivery of DNA/liposomal complex is that the distribution of the fluid within the injured area can be visualized and documented on videotape. Apparently, the release of small bubbles from dissolved air in fluids at room temperature injected into tissue causes the area perfused to become hyperechoic. This allows the radiologist to observe the regions of the nodule being injected.

State-of-the art facilities exist at Mayo for carrying out sonographically-directed biopsy and delivery of materials at deep sites in the body. Dr. Charboneau and his colleagues have now used 95% ethanol as an ablative agent in over 20 patients with hepatic tumors who were not candidates for other surgical or medical therapy. This has involved injecting solitary or, occasionally, up to three intrahepatic masses 5 cm or less in diameter. In most instances, 2–8 mL of ethanol is injected into multiple regions of an individual mass. The procedure is done with local anesthesia in an outpatient setting, and has been repeated 2 to 5 times over several weeks, provided regression is induced and appears to be continuing. For the in vivo gene transfer study, patients will be admitted to the Mayo General Clinical Research Center (GCRC) in Saint Marys Hospital. There is an ultrasound suite with its own holding area two floors removed from the GCRC. Invasive procedures in the ultrasound suite are carried out under conditions in which a general surgeon is immediately available in the extremely rare instance of the acute complication of bleeding. An operating room is always immediately available for such circumstances. Following the procedure, patients are kept in the diagnostic radiology area for two hours to assure stability before being transferred, in this instance, back to the GCRC. The suite contains CT scanning equipment, a procedure that can be used to visualize local bleeding.

The chemotherapy, pharmacy, and pharmacology shared resources of the Mayo Comprehensive Cancer Center are available and will be used to support this project. Darryl Grendahl, B.S. Pharm., is in charge of the Mayo Chemotherapy Shared Resource and is a member of the Department of Pharmacy, Rochester Methodist Hospital. He will assure the storage, preparation, and delivery of the final DNA liposomal product to the ultrasound suite when needed.

The design of this trial is similar to standard phase I clinical trials. HLA-B7 negative patients with colorectal cancer and hepatic metastases, two of which are clearly measurable by CT scan and one of which is appropriate for sonographically-guided biopsy and injection, will be eligible for entry into this trial. Patients will be treated on one of two schedules. On schedule A, the recombinant DNA will be administered in escalating doses to groups of 3 patients at each of 3 dose levels: 10, 50, and 250 μg. The starting dose, 10 μg, has been shown to be safe by Dr. Nabel when given by direct injection into subcutaneous metastatic melanomas in humans. On schedule A, each patient will receive only a single injection. Each of 3 patients will be observed for toxicity and response for 30 days before patients will receive DNA at the next planned higher dose, 50 μg. The same procedure will be followed at this dose level before proceeding to the final dose level, 250 μg. Schedule B will be initiated in new patients after all 3 patients given 50 μg DNA have been observed and found not to have unacceptable toxicity. On schedule B, 1 group of 3 patients will receive 10 μg DNA days 1 and 15. If at 30 days no patient has unacceptable toxicity, a second group of 3 new patients will receive 10 μg DNA on days 1, 15, and 29.

2.0 Goals

2.1 To determine safety and toxicity of direct intralesional injection of increasing amounts of a DNA/lipid mixture: VCL-1005 (HLA-B7/DMRIE/DOPE) into solid tumors in selected patients with metastatic colorectal adenocarcinoma. Escalating treatment regimens will be used and tumor growth evaluated.

2.2 To measure the cytotoxic T-cell activity directed towards antigens on tumor cells other than HLA-B7.

2.3 To measure humoral and cellular immune responses to HLA-B7.

2.4 To confirm expression in vivo of the HLA-B7 gene in the tumor cells.

2.5 To characterize the clinical response to escalating doses of the study drug by assessing the size of the injected tumor and of other tumor masses that may be present.

3.0 Patient Eligibility

3.1 Required characteristics—Prior to discussing protocol entry with the patient, call the Randomization Center to insure that a place on the protocol is open to the patient.

3.11 Must have histologically-confirmed metastases from primary adenocarcinoma taking origin in the colon or rectum and beyond surgical cure.

3.12 Must have at least two liver metastases that are clearly measurable in two dimensions on CT scan, one of which must measure at least 2.0 cm in greatest diameter and would be accessible for intralesional injection.

3.13 Must have had either prior standard therapies for their disease and have become unresponsive to them or have made the decision that other therapy would not be of any benefit.

3.14 Must be ≥18 years old.

3.15 The following laboratory values (≤10 days of registration):
- — white blood count > 3,000.
- — platelet count > 100,000.
- — hemoglobin > 9 g/dL.
- — prothrombin time ≤1 second above control.
- — serum creatinine <125% of upper limit of normal.
- — direct serum bilirubin normal.
- — SGOT and alkaline phosphatase <3 × above the upper limit of normal.

3.16 Must have a baseline Karnofsky Performance Scale (KPS) score of at least 70 (ECOG 0,1) (see Appendix I).

3.17 Estimated life expectancy of at least 12 weeks.

3.18 HLA typing must indicate that the patient is HLA-B7 negative.

3.19a Must be HBsAg and anti-HIV antibody negative since positivity for either disease may be associated with reduced immune competence.

3.19a Women/men of reproductive potential agree to use effective contraception.

3.19c Patients must be immunocompetent by having at least 2/6 positive skin tests on the anergy panel and by having a PHA lymphocyte response in the normal range.

3.2 Contraindications

3.21 Active autoimmune disease.

3.22 Hepatitis (acute or chronic active).

3.23 Active infection.

3.24 The following therapies within the past three weeks:
- — radiation,
- — chemotherapy,
- — steroidal.
- — surgery within past 2 weeks.

3.25 Diabetes mellitus not controlled by medical treatment.

3.26 Psychiatric illness that may make compliance to the clinical protocol unmanageable or may compromise the patient's ability to give informed consent.

3.27 Immunosuppressive therapy and any other experimental therapy.

4.0 Test Schedule

Tests and procedures	*<10 Days prior to registration*	*Days 2–10 of each scheduled treatment*	*During course of Rx schedule*			*Follow-up: At 8 wks post Rx, and q 8 wks until PROG, then q 8–12 wks*
			A days 15,29	*B_1 days 15,29,43*	*B_2 days 15,29,43,57*	
History and exam, ht, wt, PS	X	X	X	X	X	X
Vital signs (pulse, blood pressure, temperature)		X	X[1]	X[1]	X[1]	
Hematology group[R] WBC, HgB, PLT	X	X	X	X	X	X
Chemistry group[R] (SGOT, alk. phos., dir. and t. bili. creat.)	X	X	X	X	X	X
PT, PTT[R]	X	X	X[2]	X[2]	X[2]	
Electrocardiogram	X					
Chest x-ray	X					
CT scan (tumor meas.) & ultrasound[R]	X		X[2]	X[2]	X[2]	X
Tumor biopsy guided by hepatic ultrasound[R]	Day 1 prior to injection		X	29,43	43,57	
Urinalysis[R]	X		29	29	29	8 wks post Rx
Hb Ag, HIV titer[R]	X					
HLA-B7 phenotype[R]	X					
Assay for cytotoxic T-cells and antibody[3,R]			X[2]	X[2]	X[2]	X
Peripheral blood sample[R]	X[4]		X[4]	X[4]	X[4]	X[4]
Antinuclear antibodies[R]	X		29	29	29,57	
Serum preg. test[5]	X					
Lymphocyte blastogenesis[R]	X					
Anergy panel[R]	X					

[1] Prior to each biopsy and treatment and then every 15 minutes until stable.

[2] Biopsy and treatment days only.

[3] Biopsy tissue will be subdivided as per Section 17.2 and federal expressed priority overnight Monday–Wednesday (a.m.). Extra tissue will be sent to Dr. P.C. Roche, Immunohistochemical Core Laboratory, Hilton Building.

[4] Six 15 mL heparinized tubes, each containing 15 mL blood, will be federal expressed priority overnight on wet ice.

[5] For women of childbearing potential only. Must be done within 7 days prior to registration.

[R] Research funded (see Section 19.2).

5.0 Stratification Factors—None.

6.0 Registration/Randomization Procedures

6.1 Prior to discussing protocol entry with the patient, call the Randomization Center to insure that a place on the protocol is open to the patient.

6.2 To register a patient, call (4-2753) or fax (4-0885) the Randomization Center between 8 a.m. and 5 p.m. central time Monday through Friday.

6.3 A signed HHS 596 form must be on file in the Randomization Center before an institution may register any patients.

6.4 Patient eligibility and the existence of a signed consent form will be checked by Randomization Center personnel before a patient will be registered into this study. Dose level will be given.

6.5 Treatment on this protocol must be given at the Mayo Clinic-Rochester on an inpatient basis under the supervision of a medical oncologist. Actual intralesional injection of tumor will be carried out by Dr. J. William Charboneau or Dr. Carl Reading, Department of Diagnostic Radiology.

6.6 Pretreatment tests must be completed within the guidelines specified on the test schedule.

7.0 Protocol Treatment

7.1 Patients will be admitted to the General Cancer Research Center (GCRC) at Saint Marys Hospital prior to each intralesional injection.

7.2 Identification and localization of a specific liver metastasis for injection using hepatic ultrasound meeting the criteria stated in Section 3.0 will be carried out by Drs. Charboneau or Reading.

7.3 The appropriate volume of DNA/lipid mixture prepared in Saint Marys Pharmacy will be injected into the preselected hepatic metastasis on day 1. The injection is done by the diagnostic radiologist using standard procedures in the Diagnostic Radiology Suite at Saint Marys Hospital. The needle is inserted under direct sonographic visualization. A volume of between 1.0 and 4.0 mL DNA/lipid complex will be used at all DNA concentrations.

7.4 Amounts of DNA to be injected:

Schedule A

Group	*Number of Patients*	*μg of DNA*
A-1	3	10 μg day 1 only
A-2	3	50 μg day 1 only
A-3	3	250 μg day 1 only

Schedule B
(Will be initiated after safety of schedule A at 50 μg has been established)

Group	*Number of patients*	*μg of DNA*
B-1	3	10 μg days 1 and 15
B-2	3	10 μg days 1, 15, and 29

7.5 Criteria for retreatment: Patients with a partial response at 4 weeks after the last injection of their initial course may receive an additional course of treatment identical to the first treatment, i.e., on Schedule A, at 4 weeks a responding patient may receive a second injection at the same dose initially given; on Schedule B, 4 weeks after two injections (B-1) and 4 weeks after three injections (B-2), responding patients may receive a second course identical to their first course. The possibility for further injections if tumor regression occurs, will be determined on a case-by-case compassionate basis by the investigator in consultation with the patient/subject and the sponsor.

7.6 The primary objectives of this investigation are to determine the feasibility and safety of intralesional injections of DNA/lipids into liver metastases. Patients will be entered in groups of 3 at each dose level and observed for at least 4 weeks before entry of additional patients.

Number of patients with ≥grade 3 toxicity (World Health Organization [WHO] Appendix II)	*Instructions for entry of additional patients*
0	Escalate to next volume level.
1	Enter 3 more patients at same volume level.
2 or 3	Discontinue further patient entry at that level and enter 3 more patients at the previous level.

8.0 Treatment Modification Based on Toxicity

Toxicity	*Change*
SGOT or alk phos >3 × UNL *and* ≥50% ↑ compared to preRx baseline	
Direct bili ≥0.2 mg/dL above UNL	
Other Rx complications or medical conditions which substantially ↑ risk to patient	No further Rx
Symptomatic tumor PROG requiring other Rx e.g. RT or chemo	

9.0 Ancillary Treatment

Symptomatic care may be given as required with medications such as antiemetics and analgesics. However, administration of corticosteroids will require that the patient be removed from study.

10.0 Toxicity Monitoring and Adverse Reaction Reporting

10.1 Patients will be monitored and questioned at every visit (see Schedule of Events) regarding the occurrence and nature of any adverse experiences. An event is any change is the physiological or psychological state other than the primary condition that qualifies the patient for this study.

10.2 The investigator must report to Mr. Steven Kradjian (619/453-9900, available 24 hours) upon occurrence of any life-threatening events (grade IV) that may be attributable to administration of the study drug, all fatal events, or the first occurrence of any previously known clinical event (regardless of grade). A written report is to follow within 3 working days to:

Steven A. Kradjian, Director
Regulatory Affairs
Vical Inc.
9373 Towne Centre Drive, Suite 100
San Diego, CA 92121

10.3 Patients will be taken off study immediately if:

- — Unacceptable toxicity (grade III or IV) develops and is not easily corrected (see Appendix II).
- — Development of progressive disease (see definition) requiring the institution of alternative treatments such as radiation, surgery, or other drug therapy.
- — If the investigator believes that the patient's best interest requires a change of therapy.
- — At the patient's or guardian's request.

10.4 Toxicities to be graded at each evaluation and pretreatment symptoms/conditions to be evaluated at baseline per WHO criteria (Appendix II):

Toxicity/symptoms	*Baseline*	*Each evaluation*
Fatigue	X	X
Weight loss/anorexia	X	X
Nausea	X	X
Vomiting	X	X
Hemorrhage		X
Infection		X
Liver (SGOT, alk phos. and total bilirubin)		X

11.0 Treatment Evaluation

11.1 Patients will be evaluated by a Mayo Clinic medical oncologist at least every 4 weeks while receiving treatment, every 8 weeks until there is evidence of tumor progression, and then at 8–12 week intervals thereafter as long as the patient is able to return to the Mayo Clinic.

11.2 Evaluation of tumor regression is a secondary endpoint in this clinical trial. The following criteria will apply to measurable indicator lesions within the liver or for any measurable extrahepatic lesions that may be present.

11.3 Minimum size requirements for measurable indicator lesions

11.31 Tumor masses with clearly defined bidimensional measurements.

The minimum size of the indicator lesion depends on the method of measurement as follows:

Method of measurement	*Minimum size of largest tumor diameter*
Physical examination or chest x-ray (discrete lesion that can be measured with calipers)	1.0 cm
CT scan*	3.0 cm

**Note:* Special attention should be paid to identify the specific lesion being measured, and to define an anatomic landmark to identify the level of the cross-sectional image to facilitate serial measurements. Lesions 2.0–3.0 cm in diameter may be used as indicator lesions if serial images are obtained at 0.5 cm intervals through the tumor.

11.4 Criteria for a partial response (PR)

11.4.1 Bidimensional indicator lesion (s)

≥50% reduction in the sum of the products of the largest perpendicular diameters of the indicator lesion(s), single or multiple sites, chosen prior to therapy. Response satus will be determined separately for the injected tumor nodule and other measurable lesions that are not injected.

11.5 Criteria for complete response (CR)

Total disappearance of all evidence of tumor.

For a patient to qualify for complete response or partial response, none of the factors constituting progression may be present (see below).

11.6 Criteria for progression (PROG)

Tumor progression will be declared if one or more of the following criteria are met.

11.6.1 Appearance of new lesion(s).

11.6.2 Increase in tumor size.

11.6.2.1 Patients with measurable indicator lesion(s) who have met the criteria for partial response:
Significant increase in the size of indicator lesion(s) compared to the *smallest* measurements while on study.
Progression will be declared when the indicator lesion has increased in size from the smallest measurement by at least 50% of the decrease in size between pretreatment measurements and smallest measurement at the point of maximum tumor reduction.

11.6.2.2 Patients with measurable indicator lesion(s) who have met the criteria for complete response:
Progression will be declared if there is reappearance of any tumor.

11.6.2.3 Patients with measurable indicator lesion(s) compared to *pretreatment* measurements.

11.6.3 Significant clinical deterioration that cannot be attributed to treatment or other medical conditions.

11.6.3.1 Weight loss >5% body weight.

11.6.3.2 Worsening of tumor-related symptoms.

11.6.3.3 Decline in performance status >1 level on ECOG scale.

11.7 Criteria for stable (STAB)

Failure to meet the criteria for complete response, partial response, regression, or progression.

12.0 Descriptive Factors—None.

13.0 Treatment/Follow-up Decision at Evaluation of Patient

Patients meeting the criteria for partial response at 4 weeks after their single injection on Schedule A or after their last injection on Schedule B may be retreated once, on a case-by-case basis.

14.0 Pharmacologic/Immunologic Studies

14.1 Immunochemical staining will be done in the Immunohistochemical Core Laboratory of the Mayo Cancer Center under the direction of Dr. Patrick Roche. Pre- and post-treatment tumor cells are stained with anti-HLA-B7 antibodies, ME-1, BB7.1 and GSP5.3 (G. Nabel, personal communication) to look for expression.

14.2 Presence of DNA from the HLA-B7 gene will be assessed by PCR amplification of cells obtained by biopsy of the treated site on days 15, and 29 after injection of DNA/lipid complex. Genomic DNA is isolated by standard methods and a portion of the HLA-B7 gene is amplified and sequenced. Several primer sequences may be used (G. Nabel, personal communication).

14.3 Development of circulating antibodies to HLA-B7 will be evaluated. Autologous peripheral blood B lymphocytes will be EBV immortalized and subjected to *in vitro* gene transfer with the DNA/lipid complex. These autologous cell expressing the HLA-B7 gene will be used to assess the specificity of antibody response to the *in vivo* transfer of the gene. Evidence of cytolytic T-cells will be assessed if a sufficient amount of material is available for successful expansion of infiltrating T-cell population from cells in biopsy of the metastasis. These studies will be carried out in the Cellular Immunology Laboratory under the direction of Dr. Homburger.

15.0 Drug Information

The study drug (VCL-1005) will be supplied by Vical, Inc. as four sterile vials containing (i) HLA-B7 plasmid DNA, (ii) DMRIE/DOPE lipid mixture, and (iii) two vials of Lactated Ringer's Injection Vehicle. All components will be stable for at least eight weeks under recommended storage conditions (−10° to −70°C). The materials will be supplied by Vical Inc.

This study drug is composed of plasmid DNA coding for the complete human MHC HLA-B7 formulated with the cationic lipid mixture DMRIE/DOPE (lipid copmlex formulation). The DNA concentration is 1.0 mg/mL (see Investigator's Brochure for complete details of product characteristics and preparation).

DNA/lipid complexes are prepared immediately prior to administration. DNA is supplied in 1.0 mg/mL concentration in 400 μL lactated Ringer's solution. Lipid (DMRIE/DOPE) is supplied as a dried film. Each vial is reconstituted with 400 μL Lactated Ringer's solutions by vortexing until homogeneous. The contents of the lipid vial is transferred into the DNA vial and mixed well by repeated inversion. The final concentration of the HLA-B7 plasmid DNA is 500 μg/mL. The DNA/lipid complex must be prepared within six hours of administration. The amount of DNA/lipid complex injected into each tumor will be adjusted to between 1.0 and 4.0 mL with Lactated Ringer's Injection Vehicle, according to tumor size. See Investigator's Brochure for complete details of product characteristics and preparation.

Tumor lesions will be selected for treatment if they are accessible to intratumor administration by direct needle injection or intravascular catheter. These metastatic lesions will be located in the liver for colorectal adenocarcinoma. The study drug will be injected with the aid of sonographic visualization of the metastasis.

Prior to injection following placement of the needle, gentle aspiration will be applied to the syringe to ensure that no material is injected intravenously. Vital signs will be measured every 15 minutes prior to, during, and after the injection for at least two hours or until the patient is stable. If the systolic blood pressure drops below 80 mm Hg, the injection will be terminated immediately, and the patient will be closely monitored until blood pressure is normalized.

Every patient will be observed for 48 hours, and a blood sample will be obtained to check serum enzymes, blood chemistries and cell counts, and to analyze by PCR for the presence of HLA-B7 plasmid DNA in the peripheral blood. If there are no complications, the patient will be discharged. If any abnormalities appear, the patient will be closely observed. All toxicities will be graded according to the WHO recommendation (see Appendix II).

16.0 Statistical Consideration and Methodology

Descriptive statistics only will be performed due to the small number of patients.

17.0 Pathology Considerations

17.1 Patients entering this trial will already have had histologically-documented metastatic colon carcinoma. Liver biopsy is carried out as part of this protocol including the initial one to demonstrate unequivocally by histologic examination of a frozen section that the metastasis selected for injection of the DNA/liposome complex is, in fact, metastatatic cancer. Biopsy samples are never to be placed in fixative. Jill Piens, R.N. (507/284-4911 or pager 4-6362) to call Dr. J.S. Kovach's laboratory and state that a biopsy sample on this

immunotherapy protocol has been obtained and is being held in a dry sterile container placed on ice to be picked up.

17.2 For biopsies in which a core of tumor has been obtained, the sample will be subdivided (minimum 1 core [1–2 cm × 1mm], snap frozen, LN2, cryovial or 2 cores, one on wet ice, one snap frozen or 3 cores, one on wet ice, two snap frozen). The remainder may be subjected to tissue disruption in an attempt to obtain and expand T-cells present in the tissue.

18.0 Records and Data Collection Procedures

18.1 Mayo Oncology Records

18.11 The Oncology Records will be utilized for patients. Data will be entered into the computer within 3 weeks after each evaluation of the patient. After the patient goes off treatment, follow-up information will be collected and entered into the computer every 3 months.

18.12 Pathology diagnosis made by frozen section is reported in a conventional way with a record being placed in the patient's permanent medical record.

18.13. Data from the Cellular Immunology Laboratory may be entered into their computer using the patient's clinic number as a key. Summary reports of samples received will be sent to the principal investigator monthly.

18.14 Patient follow-up for survival will continue at least annually for the patient's lifetime.

18.2 Vical records

18.21 Case report forms, regulatory documents and study source documents will be collected and reviewed in conformance with FDA Good Clinical Practices.

19.0 Budget

19.1 Costs charged to patient: Routine tests to monitor the patient's malignant disease and assess response to therapy will be charged to the patient or third party carrier.

19.2 Tests to be research funded: Charges for intralesional administration of the test agent and biopsy will not be charged to the patient. Research expenses will be supplied by Vical, Inc. (see attached budget).

19.3 Other budget concerns: Patients will be admitted to the GCRC at Saint Marys Hospital. No charge will be made for their hospital room.

GCRC Pharmacy charges:

$57/patient/treatment

15 patients × 3 treatments = 45 × $57 = $2,565

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Principal Investigator: Rubin, J., M.D.
Funding Source: Vical Inc.
Title: Phase I Study of Immunotherapy of Advanced Colorectal Carcino by In Vitro Gene Transfer

PERSONNEL	TITLE	% EFFORT	TOTAL AMOUNT
Rubin, J., M.D.	Principal Investigator	10%	
Piens, J., R.N.	Study Coordinator	25%	
Garcia, J.	Data Manager	25%	
	Total Salaries		15,555
	Benefits @ 22.7% of salaries		3,531
	Total Personnel		19,086

PATIENT COSTS

** For All Listed Procedures: 15 patients	
Pretreatment	
Ultrasound (#7583 @ $336.51*)	5,048
Computer Assisted Tomography (CT)-abdomen w/ contrast (#74160 @ $644.95)	9,674
Venipuncture (#8200 @ $10.21*)	153
Chemistry Group (#9093 @ $48.05*)	721
Hematology (#9109 @ $12.06*)	181
UA (#9308-1 @ $18.66*)	280
HLA-B7 Phenotyping (#9648 @ $135.00)	2,025
HIV Antibody Screen (#9333 @ $70.91)	1,064
Hb-A5 Antibody Screen (#8341 @ $47.67)	715
Treatment (Day 1 for 9 patients; Days 1 and 15 for 3 patients; Days 1, 15 and 30 for 3 patients)	
Ultrasonic guided biopsy (#7599 @ $501.16*)	12,028
Venipuncture (#8200 @ $10.21*)	245
Chemistry Group (#9093 @ $48.05*)	1,153
Hematology (#9109 @ $12.06*)	289
UA (#9308-1 @ $18.66*)	448
PT (#9236 @ $13.20*)	317
PTT (#9058 @ $18.39*)	441
Days 3, 15, 30 and 60 for 15 patients	
Ultrasonic guided biopsy (#7599 @ $501.16*)	30,070
Venipuncture (#8200 @ $10.21*)	613
Chemistry Group (#9093 @ $48.05*)	2,883
Hematology (#9109 @ $12.06*)	724
UA (#9308-1 @ $18.66*)	1,120
PT (#9236 @ $13.20*)	792
PTT (#9058 @ $18.39*)	1,103
Days 15, 30 and 60 for 15 patients	
Computer Assisted Tomography (CT)-abdomen w/ contrast (#74160 @ $644.95)	29,023
Ultrasounic guided biopsy (#7599 @ $501.16*)	22,552
HLA Serological screening @ $120 - Days 15, 30, 60 & 90 for 15 patients	7,200
Total Patients Costs	130,862
TOTAL DIRECT COST	149,948
INDIRECT COST @ 53% MTDC	10,116
TOTAL COST	160,064

Rubin J. - Vical, Inc. (Oncology)
MAP: 9/27/93 Revised: 1/21/94

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Title: Phase I Study of Immunotherapy of Hepatic Metastases of Colorectal Carcinoma by Direct Gene Transfer

Participants: Joseph Rubin, M.D.; J. William Carboneau, M.D.; Carl Reading, M.D.; John S. Kovach, M.D.

SUGGESTED PATIENT CONSENT FORM

FEDERAL REGULATIONS REQUIRE WRITTEN INFORMED CONSENT FROM PARTICIPANTS PRIOR TO PARTICIPATION IN A RESEARCH STUDY SO THAT THEY CAN KNOW THE NATURE AND RISKS OF PARTICIPATION AND CAN DECIDE TO PARTICIPATE OR NOT TO PARTICIPATE IN A FREE AND INFORMED MANNER. YOU ARE ASKED TO READ THE FOLLOWING MATERIAL TO ENSURE THAT YOU ARE INFORMED OF THE NATURE OF THIS RESEARCH STUDY AND OF HOW YOU WILL PARTICIPATE IN IT IF YOU CONSENT TO DO SO. SIGNING THIS FORM WILL INDICATE THAT YOU HAVE BEEN SO INFORMED AND THAT YOU GIVE YOUR CONSENT.

The purpose of this study is to determine the safety and optimal dose of a DNA (deoxyribonucleic acid)/liposome compound when injected directly into colorectal cancers which have spread to the liver. The DNA/liposome complex contains a human gene (referred to as HLA-B7) which is known to enhance rejection of foreign cells by the immune system in individuals who do not carry this gene naturally. The goal of this study is to transfer this gene to the cancer cells in the liver, thereby making them (and possibly any cancer cells elsewhere in the body) susceptible to destruction by the normal immune cells (cytotoxic lymphocytes) of the patient. The chance of benefit from this experimental therapy cannot be predicted.

Participants in this study will be admitted to the General Clinical Research Center (GCRC) at Saint Marys Hospital. Up to four direct injections of the DNA/liposome complex into a single liver tumor will be performed in the Diagnostic Radiology Department at Saint Marys Hospital using an ultrasound technique (sound waves) to directly visualize the tumor. A biopsy of the tumor will be obtained immediately prior to the first injection. The injections will be made under sterile conditions after providing a local anesthetic (xylocaine), and multiple areas within a single nodule will be injected up to 4 times. The duration of this procedure is usually 30 minutes. Participants will be observed for 24–48 hours in the GCRC following the injection. Biopsies of the liver nodule will be repeated at Saint Marys Hospital two weeks and four weeks after injection of the tumor. If there is evidence that the tumor is shrinking four weeks following injection, the participant may receive one or two additional injections into the liver at 4-week intervals. These additional injections will be considered on a case-by-case basis only, following consultation with the patient-subject, physician-researchers, and the sponsor. If uninjected tumor nodules elsewhere in the body also shrink, a needle biopsy of these tumors may also be done to study the mechanism of tumor destruction. Blood samples for research purposes will be obtained prior to tumor injection at 1, 2, 4, 8 weeks, then every 3 months for 1 year. The total volume of blood drawn for research purposes will be less than 500 mL (one pint). The investigators will stay in touch with study participants after completion of this study to keep track of their progress over their life. Permission for an autopsy will be requested if the patient expires.

No side effects other than local inflammation have been observed in preliminary studies of direct injection of a similar DNA/liposome complex into skin tumors in patients with malignant melanoma. There is a theoretical possibility that inflammation of normal body tissue producing symptoms such as joint pain, skin rash, or damage to the kidneys could occur in response to transfer of the HLA-B7 gene to the tumor. There is also the potential risk of damage to the liver if severe inflammation occurs around the injected liver tumor. Bleeding and infection are rare complications associated with needle biopsies of tumors in the liver or elsewhere within the body. All care will be taken to minimize the risk of complication, although the nature and severity of side effects is unpredictable.

Although there is no evidence that the gene transferred to the tumor cells can also enter egg cells or sperm cells, this is a theoretical possibility. Therefore, this study may be hazardous to an unborn child. There is no information as to whether there are significant risks to a fetus carried by a mother who is exposed to the DNA/liposome complex. Therefore, only men and women who agree to use effective means of contraception, or are unable to have children, may participate in this study.

Alternative interventions for this type of malignant disease in the liver spreading from a cancer of the colon or rectum would consist of investigative chemotherapy or symptomatic care. There is no known cure for this condition. A physician will discuss these alternative approaches in detail as they may relate to the patient's decision to participate.

Participants in this investigational program, as in most cancer research, undergo periodic monitoring for signs of change in the condition of their tumors, for any toxic effects of the research intervention, and to allow the physician-researchers to adjust what they are doing. Such monitoring includes physical examinations and studies such as x-rays, scans, and blood tests. All such procedures carried out for research purposes, as well as room and board and nursing charges at the General Clinical Research Center, will not be billed to patient-subjects in this study. Procedures that are solely for treatment purposes, as well as pharmacy charges and other ancillary expenses such as oral

medications prescribed at the time of discharge, will be billed to the patient-subjects and/or their insurers.

The principal investigator, *Dr. Joseph Rubin*, or a co-investigator, telephone *507/284-4718*, may be contacted at any time if the participant has any questions regarding this study, if further information about the nature and conduct of the study is required, or if any problems result from participation, including a research-related illness. Participation in this research study is voluntary and the participant may refuse to enter the study or may discontinue participation in the study at any time without jeopardizing present or future medical care and treatment or other benefits to which the participant is entitled. The investigators may discontinue the participant's study involvement at any time if it is felt to be in the participant's best interest, if the participant does not comply with the study requirements, or if the study is stopped. Participants will be informed of significant new information, and of any changes in the nature of the study or in the procedures described if they occur.

No commitment is made to provide free medical care or compensation during participation in this study. Medical services not directly related to the study will be offered at the usual charge. Further information concerning policies in this regard or information about the conduct of this study or the rights of research subjects, may be obtained from *(IRB or Legal Department)*, telephone *507/284-2511*.

Although data from this study may be published, confidentiality of information concerning participants will be maintained. Names of participants or material identifying participants will not be released without written permission except as such release is required by law. Medical records related to this study may be made available to the National Institutes of Health, Food and Drug Administration, or the sponsor as provided in federal regulations.

I HAVE HAD AN OPPORTUNITY TO HAVE MY QUESTIONS ANSWERED. A COPY OF THIS FORM HAS BEEN GIVEN TO ME. I AGREE TO PARTICIPATE IN THIS MEDICAL RESEARCH STUDY UNDER THE DIRECTION OF THE PRINCIPAL INVESTIGATOR AS LISTED ABOVE.

(Date)	(Signature of Participant)
(Date)	(Signature of Investigator Obtaining Consent)

APPENDIX I. KARNOFSKY PERFORMANCE SCALE

Activity status	*Point*	*Description*
Normal Activity	100	Normal, with no complaints or evidence of disease
	90	Able to carry on normal activity but with minor signs or symptoms of disease present
	80	Normal activity but requiring effort; signs and symptoms of disease more prominent
Self-care	70	Able to care for self, but unable to work or carry on other normal activities
	60	Able to care for most needs but requires occasional assistance
	50	Considerable assistance required, along with frequent medical care; some self-care still possible
Incapacitated	40	Disabled and requiring special care and assistance
	30	Severely disabled; hospitalization required but death from disease not imminent
	20	Extremely ill, supportive treatment, hospitalized care required
	10	Imminent death
	0	Dead

APPENDIX II. WHO RECOMMENDATIONS FOR GRADING OF ACUTE AND SUB-ACUTE TOXIC EFFECTS

	Grade 0	*Grade 1*	*Grade 2*	*Grade 3*	*Grade 4*
Hematological (Adults)					
Hemoglobin	≥11.0 g/100 ml ≥100 g/l ≥6.8 mmol/l	9.5–10.9 g/100 ml 95–109 g/l 5.6–6.7 mmol/l	8.0–9.4 g/ml 50–94 g/l 4.95–5.8 mmol/l	6.5–7.9 g/100 ml 65–79 g/l 4.0–4.9 mmol/l	<6.5 g/100 ml <65 g/l <4.0 mmol/l
Leukocytes (1000/mm^3)	≥4.0	3.0–3.9	2.0–2.9	1.0–1.9	1.0
Granulocytes (1000/mm^3)	≥2.0	1.5–1.9	1.0–1.4	0.5–0.9	<0.5
Platelets (1000/mm^3)	>100	75–99	50–74	26–49	<25
Hemorrhage	None	Petechiae	Mild blood loss	Gross blood loss	Debilitating blood loss
Methemoglobin[a]	0–2.0%	2.1–10.0%	10.1–20.0%	20.1–30.0%	>30%
Gastrointestinal					
Bilirubin	≤1.25 × N[b]	1.26–2.5 × N[b]	2.6–5 × N[b]	5.1–1.0 × N[b]	>10 × N[b]
Transaminases (SGOT/SGPT)	≤1.25 × N[b]	1.26–2.5 × N[b]	2.6–5 × N[b]	5.1–10 × N[b]	>10 × N[b]
Alkaline phosphatase	≤1.25 × N[b]	1.26–2.5 × N[b]	2.6–5 × N[b]	5.1–10 × N[b]	>10 × N[b]
Oral	No change	Soreness/erythema	Erythema, ulcers; can eat solids	Ulcers; requires liquid diet only	Alimentation not possible
Nausea/vomiting	None	Nausea	Transient vomiting	Vomiting requiring therapy	Intractable vomiting
Diarrhea	None	Transient, <2 days	Tolerable, but >2 days	Intolerable, requiring therapy	Hemorrhagic dehydration
Renal					
Blood urea nitrogen or Blood urea creatinine	≤1.25 × N[a]	1.26–2.5 × N[a] 1+	2.6–5 × N[a] 2–3+	5–10 × N[a] 4+	>10 × N[a]
Proteinuria	No change	<0.3 g% <3 g/l	0.3–1.0% <3–10 g/l	>1.0 g% >10 g/l	Nephrotic syndrome
Hematuria	No change	Microscopic	Gross	Gross + clots	Obstructive uropathy
Pulmonary	No change	Mild symptoms	Exertional dyspnea	Dyspnea at rest	Complete bed rest required
Fever with drug	None	Fever <38°C	Fever 38°C–40°C	Fever >40°C	Fever with hypotension
Allergic	No change	Edema	Bronchospasm; no parental therapy needed	Bronchospasm; parental therapy required	Anaphylaxis